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Research article

## Effects of shade on the development and sugar metabolism of coffee (*Coffea arabica* L.) fruits

Clara Geromel <sup>a</sup>, Lúcia Pires Ferreira <sup>b</sup>, Fabrice Davrieux <sup>d</sup>, Bernard Guyot <sup>d</sup>,  
Fabienne Ribeyre <sup>d</sup>, Maria Brígida dos Santos Scholz <sup>b</sup>, Luiz Filipe Protasio Pereira <sup>c</sup>,  
Philippe Vaast <sup>f</sup>, David Pot <sup>b,e</sup>, Thierry Leroy <sup>e</sup>, Armando Androcioni Filho <sup>b</sup>,  
Luiz Gonzaga Esteves Vieira <sup>b</sup>, Paulo Mazzafera <sup>a</sup>, Pierre Marraccini <sup>b,e,\*</sup>

<sup>a</sup> Departamento de Fisiologia Vegetal—IB, UNICAMP, CP 6109, 13083-970 Campinas, SP, Brazil<sup>b</sup> IAPAR, LBI-AMG, CP 481, 86001-970 Londrina, PR, Brazil<sup>c</sup> EMBRAPA Café—LBI, CP 481, 86047-902 Londrina, PR, Brazil<sup>d</sup> CIRAD-UMR Qualisud, TA B-95/16, 73 rue JF Breton, 34398 Montpellier, France<sup>e</sup> CIRAD-UMR DAP, Avenue d'Agropolis, 34398 Montpellier, France<sup>f</sup> CIRAD-UPR 80, CATIE, Apdo 3, Turrialba, Costa Rica

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### Abstract

Coffee fruits grown in shade are characterized by larger bean size than those grown under full-sun conditions. The present study assessed the effects of shade on bean characteristics and sugar metabolism by analyzing tissue development, sugar contents, activities of sucrose metabolizing enzymes and expression of sucrose synthase-encoding genes in fruits of coffee (*Coffea arabica* L.) plants submitted to full-sun (FS) and shade (SH) conditions. Evolution of tissue fresh weights measured in fruits collected regularly from flowering to maturation indicated that this increase is due to greater development of the perisperm tissue in the shade. The effects of light regime on sucrose and reducing sugar (glucose and fructose) contents were studied in fresh and dry coffee beans. Shade led to a significant reduction in sucrose content and to an increase in reducing sugars. In pericarp and perisperm tissues, higher activities of sucrose synthase (EC 2.4.1.13) and sucrose-phosphate synthase (SPS; EC 2.4.1.14) were detected at maturation in the shade compared with full sun. These two enzymes also had higher peaks of activities in developing endosperm under shade than in full sun. It was also noted that shade modified the expression of SUS-encoding genes in coffee beans; *CaSUS2* gene transcripts levels were higher in SH than in FS. As no sucrose increase accompanied these changes, this suggests that sucrose metabolism was redirected to other metabolic pathways that need to be identified.

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**Keywords:** Bean composition; *Coffea arabica*; Fruit development; Gene expression; Shade; Sucrose synthase; Sugar metabolism

### 1. Introduction

Sucrose represents the major transport form of photosynthetically assimilated carbon in plants and its metabolism plays

a key role, particularly in sink tissues such as fruits [23]. The key enzymes of sucrose metabolism are invertases (EC 3.2.1.26,  $\beta$ -fructosidase,  $\beta$ -fructofuranosidase), sucrose phosphate synthase (SPS; UDP-glucose: D-fructose 6-phosphate 2-glucosyl-transferase) and sucrose synthase (SUS; UDP-glucose: D-fructose 2-glucosyl-transferase). Invertases catalyze the irreversible hydrolysis of sucrose to glucose and fructose; they are also involved in plant responses to environmental stimuli [34]. In contrast, SPS functions mainly in sucrose synthesis (UDP-glucose + fructose-P  $\rightarrow$  sucrose-P + UDP),

**Abbreviations:** DAF, days after flowering; FS, full sun; NIRS, near infrared reflectance spectroscopy; SH, shade.

\* Corresponding author at present address: EMBRAPA—Center of Genetic Resources and Biotechnology, Parque Estação Biológica, CP 02372, 70770-900 Brasília (DF), Brazil. Tel.: +55 61 3448 4795; fax: +55 61 3340 3624.

E-mail address: [marraccini@cirad.fr](mailto:marraccini@cirad.fr) (P. Marraccini).

whereas SUS catalyses a reversible reaction (UDP-glucose + fructose  $\leftrightarrow$  sucrose + UDP). Both enzymes are thought to play a major role in sucrose partitioning for energy purposes as well as in metabolic, structural and storage functions in plant cells [39]. The sucrolytic activity of SUS is also linked to cell wall biosynthesis by providing UDP-glucose for the cellulose synthase complex [3] and substrates for starch synthesis in sink organs [18]. In contrast, an increase in SUS activity associated with sucrose accumulation was reported in several fruits such as peach, citrus and muskmelon [19,20,25,26].

In coffee and many other plants, sucrose is essential for the control of sink-source mechanisms [11]. Sucrose is also one of the main compounds in coffee beans, varying from 5% to 9.5% of dry matter basis (dmb) in *Coffea arabica* and from 4% to 7% of dmb in *Coffea canephora* [22]. Although considerably degraded during bean roasting, sucrose remains noticeably present in roasted grains at concentrations of 0.4–2.8% dmb and is likely to contribute to beverage sweetness [17]. It is also the main contributor of reducing sugars which are implicated in Maillard reactions occurring during the roasting process [15]. The higher sucrose contents in Arabica green bean could partially explain its better cup quality [22].

Coffee beverage quality also depends on various environmental factors, including shade [4,9]. Artificial shade [1] or shade trees [5] reduce coffee fruit load through their effects on coffee physiology, such as longer internodes, fewer fruiting nodes and lower flower induction. In contrast, shade results in beans of larger size [27]. Altogether, this leads to higher coffee quality, probably by lowering tree stress, which favors slow fruit ripening and hence better bean filling [42]. The effect of shade on sucrose content in mature beans has also been documented, but remains controversial. Increased sucrose content was reported for the Catuai cultivar of *C. arabica* grown under shade conditions [17], whereas a negative relationship between sucrose and shade was observed for the Catimor cultivar of *C. arabica* [42]. Sucrose content in coffee beans also seems to depend on the particular *C. arabica* cultivar since a positive correlation was observed between altitude and sucrose for Catuai but not for Bourbon [17]. Sucrose accumulation in developing coffee fruit was first studied in *C. canephora* by Guyot et al. [16] who showed that bean sucrose content varied with the maturation degree and ripening speed; sucrose content was found to increase from green to red-violet cherries and had lower values in precocious fruits than in late fruits. More recently, sucrose metabolism and the role of sucrose synthase were investigated during development in the fruit tissues (pericarp, perisperm and endosperm) of *C. arabica* L. cv. IAPAR 59 [12], a “Sarchimor” cultivar derived from a cross between Villa Sarchi (CIFC 971/10) cultivar and Timor hybrid (CIFC 832/2) [37]. This study highlighted the importance of the pericarp and perisperm tissues in providing sugar precursors for endosperm development and identified SUS as one of the main enzymes responsible for sucrose accumulation in mature beans.

Despite the rapid development of coffee specialty markets, in-depth studies of shade effects on coffee bean metabolism

are yet to be undertaken. The aim of this work was to initiate such analyses on tissue evolution, sucrose content, activities of sucrose-metabolizing enzymes and gene expression in developing fruits of plants grown under shade (SH) and full-sun (FS) conditions. Using the same cultivar (*C. arabica* L. cv. IAPAR 59) as that studied in our previous work on sucrose metabolism in developing fruits grown under FS field conditions [12], the present study is the first to investigate this with respect to sucrose metabolism in developing fruits of plants grown under SH and FS conditions. The results presented here are of general interest, particularly for distinguishing shade effects from other factors.

## 2. Materials and methods

### 2.1. Plant material

Fifteen-year-old plants of *Coffea arabica* L. cv. IAPAR 59 [37] were cultivated (2 m between rows  $\times$  1 m within rows) at the experimental station of the Agronomic Institute of Paraná State (Iapar, Londrina, Brazil) in FS condition without irrigation. For the shade plot (SH), light was artificially reduced using a black net (30 m  $\times$  20 m) covering about 150 plants and allowing 50% of the photosynthetic photon flux density (PPFD). The net was installed over the plants 1 month after the main flowering, which occurred between 21 and 24 September, 2003. Weeds were controlled manually and plants were equally fertilized in both plots.

### 2.2. Fruit sampling and processing

In FS and SH conditions, fruits were harvested every four weeks after flowering until cherry fruit maturity. For the shade plot, fruits from plants of border lines were not collected. After collection, fruits were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . To ensure developmental synchrony of harvested fruits, cross sections were made to visually inspect the tissues (pericarp, perisperm and endosperm) that were separated and frozen for further analyses. The mature stage corresponded to cherry fruits with red pericarp turning to purple and containing hard white endosperm with the remaining perisperm forming a thin brown pellicle surrounding the endosperm.

Sugar quantification and enzymatic analyses were performed using one bulked sample (100 g) of fruits from ten plants randomly chosen within the plots. For physical and biochemical analyses of dry beans, three sets (1.5 kg) of 5–7 plants each were harvested for the FS and SH plots for two different dates: 214 and 281 days after flowering (DAF). In order to compare SH and FS mature fruits, only ripe fruits were harvested (over-ripe cherries with a dried pericarp turning brown to black, were not considered). Fruits from the two harvests were then sun-dried for 15–20 days until beans reached 10–12% humidity levels. Next, dried pericarp and endocarp (parchment) were mechanically removed. Bean density was measured by weighing three sub-samples (100 beans each) and measuring water displacement (with 0.1% Tween-20) in

a 100 ml test tube. Bean size distribution was also evaluated using a series of sieves with round perforations ranging from 4.8 to 7.1 mm [35].

### 2.3. Weight measurements and tissue separation

Fresh weights (FW) were evaluated after each harvest from three samples of 30 fruits at 32 and 60 DAF and from 10 fruits at later harvests which were discarded after weighing. For tissue dissections, three samples of 10 fruits frozen at  $-80^{\circ}\text{C}$  were used and FW was measured throughout fruit development with a precision balance, except for small fruits (diameter  $<2$  mm), for which tissue separation was not possible. Pericarp and endosperm tissues were separated beginning at 60 and 120 DAF respectively, up to the mature stage. Due to its tight adhesion to the endosperm in the latest maturation stages, perisperm tissue was not separated after 175 and 231 DAF in FS and SH conditions, respectively.

### 2.4. Biochemical and enzymatic analyses

Tissues of developing fruits grown under FS and SH conditions were freeze-dried, ground with a mortar and pestle and extracted with 80% ethanol in a Polytron homogenizer using 1 ml per 300 mg of tissue. Extraction proceeded for 30 min at  $75^{\circ}\text{C}$  in cap-sealed tubes; supernatants were obtained after centrifugation. The extraction was carried out three times with the same volume of ethanol, and the combined supernatants were then used for the analysis of sugars. Sucrose content was determined according to Van Handel [43]. Levels of reducing sugars (RS: glucose and fructose) were determined according to Somogyi [38]. Lipid and trigonelline contents were measured by NIRS using dry beans (20–30 g) that had been equilibrated (for 6 days at 60% humidity and  $28^{\circ}\text{C}$ ) prior to being frozen in liquid nitrogen and ground ( $<0.5$  mm). The absorption spectrum (from 400 and 2500 nm) of the powder was measured (NIRS model 6500, NIRS System Inc., Silver Spring, MD) and compared with previously established calibration curves [9]. Data were treated by Winisi 1.5 (NIRS2 4.0) software (Intrasoft Int., Port Matilda, PA). Caffeine, chlorogenic acids (CGA), protein and tannin contents were evaluated according to the methods described in AOAC [2].

For the enzymatic analyses, proteins were extracted with 100 mM HEPES (pH 7.0), containing 2 mM  $\text{MgCl}_2$ , 10 mM 2-mercaptoethanol and 2% (w/v) ascorbic acid to avoid oxidation. The supernatant recovered by centrifugation ( $27,000 \times g$  for 20 min) was desalted on PD10 minicolumns (Amersham Biosciences) and the protein content was determined with a ready-to-use [7] reagent (BioRad). Acid invertase (AI) activity, regrouping vacuolar (soluble) and cell wall invertase (insoluble) [39] levels were assayed by incubating an aliquot of the desalted extract containing 60  $\mu\text{g}$  of protein with 25 mM sucrose in 50 mM citrate-phosphate buffer (pH 3.5) at  $37^{\circ}\text{C}$  for 1 h modified from Yelle et al. [47]. Levels of the resulting reducing sugars were determined as described before [38]. Sucrose phosphate synthase (SPS) activity was assayed in the

direction of sucrose-phosphate synthesis by incubating 60  $\mu\text{g}$  of protein with 25 mM uridine 5'-diphosphoglucose (UDPG), 25 mM fructose-6-phosphate, 30 mM glucose-6-phosphate, 20 mM phenyl- $\beta$ -glucoside in 50 mM K-phosphate buffer (pH 7.5) for 1 h at  $37^{\circ}\text{C}$ . Sucrose synthase (SUS) activity was assayed (60 min at  $30^{\circ}\text{C}$ ) in the direction of sucrose synthesis in a reaction containing 50–60  $\mu\text{g}$  of protein, 25 mM UDPG, 25 mM D-fructose and 50 mM 2-(N-morpholino)ethanesulfonic acid hydrate (MES) (pH 6.0). Enzymatic reactions were stopped by the addition of 30% (w/v) KOH and boiling for 10 min. The amounts of sucrose phosphate and sucrose formed in the SPS and SUS reactions were determined as described before [43]. Results of sugar contents and enzymatic activities are the mean  $\pm$  standard deviation (SD) of assays using three independent extractions.

### 2.5. Northern blot analysis

Total RNA was extracted from the endosperm of coffee fruits grown under FS and SH conditions, as described previously [32]. Fifteen micrograms of total RNA were fractionated on a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde in MOPS buffer, transferred to Hybond N+ membranes (Amersham Biosciences) and hybridized independently with *CaSUS1* and *CaSUS2* probes, as previously described [12]. Amounts of RNA samples loaded were controlled by the equal abundance of 18S and 26S rRNA on gels stained with ethidium bromide.

### 2.6. Statistical analysis

All statistical analyses were performed with R software [30]. Comparisons of the bean size distribution between the two light regimes were performed for each harvest date using Pearson's chi-square tests. The amounts of biochemical compounds in the dried beans expressed as a percentage of fruit dry weight were transformed as "arcsin/percentage" before statistical analysis to reach a normal distribution of residues. The effects of light treatments, harvest dates and interaction of these factors on physical properties (weight, volume and density) and fruit chemical composition of the dried beans were evaluated through a two-fixed-factor ANOVA model with interaction.

## 3. Results

### 3.1. Influence of shade on coffee fruit development

Under the present conditions, shade delayed fruit ripening by approximately one month, with a full ripening of fruits estimated at 231 DAF and 260 DAF for FS and SH conditions, respectively (Fig. 1A). For mature fruits, no difference in total fruit fresh weight (FW) was observed between FS ( $1.40 \pm 0.15$  g) and SH ( $1.39 \pm 0.11$  g) conditions. Pericarp FW followed a biphasic curve, with a first peak at 87 DAF and a second peak at 197 DAF (Fig. 1B). Pericarp FW were similar between 60 and 144 DAF for FS and SH plants

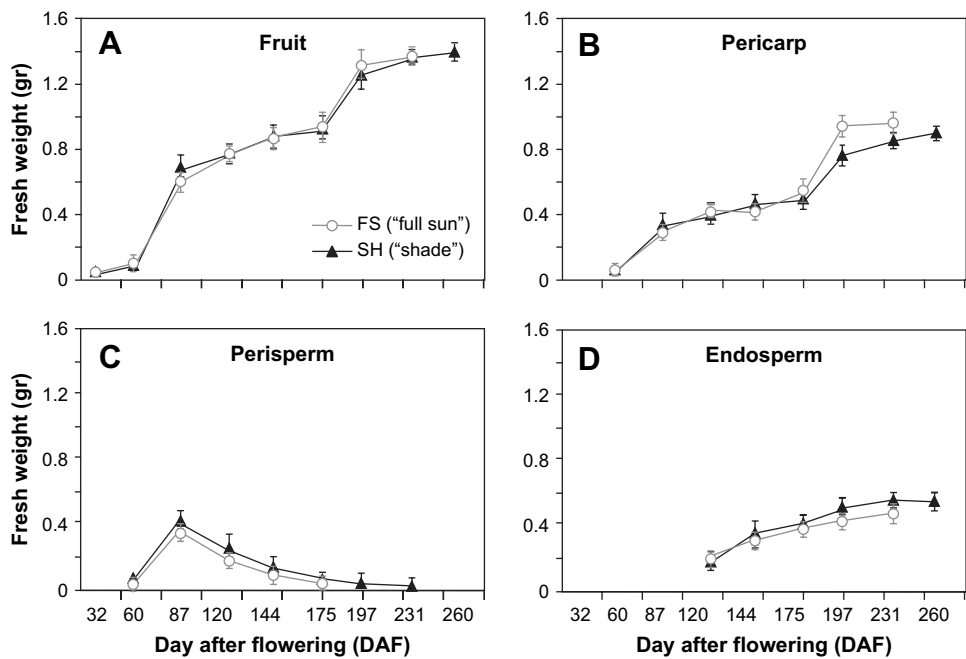


Fig. 1. Tissue evolution during the ripening process of *C. arabica* fruits grown under different light conditions. Fresh weights are given in grams for entire fruits (A), pericarp (B), perisperm (C) and endosperm (D) tissues. Open circles and closed triangles are for full-sun (FS) and shade (SH), respectively. Error bars indicate standard deviation (SD) of repetitions ( $n = 3$ ).

(Fig. 1B), whereas faster growth and higher pericarp FW were achieved in the FS compared to the SH conditions. Perisperm expansion was rapid between 60 and 87 DAF; FW was higher under SH conditions than it was in FS (Fig. 1C). After 87 DAF, the perisperm tissue disappeared gradually up to maturation, but remained separable from the endosperm for a longer period in SH fruits. The endosperm became easily detachable from the perisperm at 120 DAF. After this point, endosperm FW was always higher in SH than in FS fruits, reaching  $0.51 \pm 0.07$  g and  $0.47 \pm 0.03$  g, respectively, at harvest (Fig. 1D).

3.2. Effect of shade on bean characteristics

Bean size distribution presented significant differences between light regimes, with a higher proportion of larger beans in SH than in FS conditions, for both harvest dates (Table 1A). These values were confirmed by a chi-square test. Light condition (LC) had a significant effect on both

bean weight ( $F = 20.41$ ,  $P < 0.01$ ) and volume ( $F = 19.54$ ,  $P < 0.01$ ) (Table 1B). However, bean density was not affected by light conditions ( $F = 0.07$ ,  $P > 0.05$ ). The harvest date effect and the interaction between light conditions and harvest date effect were not significant for these traits. In contrast, bean weight ( $F = 0.36$ ,  $P > 0.05$ ), volume ( $F = 0.08$ ,  $P > 0.05$ ) and density ( $F = 0.27$ ,  $P > 0.05$ ) did not vary significantly with harvest date (HD).

3.3. Effects of shade on sugars and other biochemical compounds

Statistical analysis revealed that LC had no effects on total sugars ( $F = 4.47$ ,  $P > 0.05$ ), but significantly affected the reducing sugar ( $F = 11.05$ ,  $P < 0.05$ ) and sucrose contents ( $F = 6.59$ ,  $P < 0.05$ ) of dry beans (Table 2). While sucrose content was higher in FS than in SH conditions, the opposite was observed for reducing sugars. It is also worth noting that HD had a significant effect on RS ( $F = 14.02$ ,

Table 1A  
Size distribution (expressed in %) of mature beans of *C. arabica* L. cv. IAPAR 59 under full sun (FS) and shade (SH)

Harvest time	Bean diameter (mm)					
	<5.9		5.9–6.8		≥6.8	
	FS	SH	FS	SH	FS	SH
214 DAF	22.4 ± 5.3	13.8 ± 7.0	72.8 ± 3.4	57.6 ± 1.2	7.9 ± 4.2	27.5 ± 4.7
281 DAF	22.8 ± 4.1	12.4 ± 2.1	71.2 ± 2.9	68.2 ± 3.4	5.9 ± 1.7	19.8 ± 1.6
Chi-square test 214 DAF	$\chi^2 = 13.6321$ , $P$ value (df = 2) = 0.001096**					
Chi-square test 281 DAF	$\chi^2 = 10.5534$ , $P$ value (df = 2) = 0.005109**					

Values of Pearson's chi-square tests and the corresponding  $P$ -values are indicated.

Table 1B

Weight, volume and density values of mature beans of *C. arabica* L. cv. IAPAR 59 under full sun (FS) and shade (SH)

Harvest time	Weight (g)		Volume (cm <sup>3</sup> )		Density (g cm <sup>-3</sup> )	
	FS	SH	FS	SH	FS	SH
214 DAF	12.7 ± 0.7	13.8 ± 0.4	18.8 ± 1.0	20.5 ± 0.8	0.68 ± 0.01	0.68 ± 0.03
281 DAF	12.4 ± 0.4	13.8 ± 0.3	18.3 ± 0.6	20.7 ± 0.6	0.68 ± 0.00	0.67 ± 0.03
<i>F</i> -value LC		20.41**		19.54**		0.07 <sup>ns</sup>
<i>F</i> -value HD		0.36 <sup>ns</sup>		0.08 <sup>ns</sup>		0.27 <sup>ns</sup>
<i>F</i> -value LC × HD		0.44 <sup>ns</sup>		0.48 <sup>ns</sup>		0 <sup>ns</sup>

Data were transformed as arcsin √ percentage to perform the ANOVA analysis. *F*-values for light conditions (LC), harvest date (HD) and light condition × harvest date effects (LC × HD) are indicated. <sup>ns</sup>*P* > 0.05, \**P* < 0.05, \*\**P* < 0.01.

*P* < 0.01); a lower content was observed at 281 DAF than at 214 DAF. No LC × HD effects were observed on sugar contents. Dry bean contents of caffeine, chlorogenic acids (CGA), proteins, lipids, tannins and trigonelline were also investigated (Table 3). Light condition (LC) had a significant effect on lipids (*F* = 16.83, *P* < 0.01), where higher values were observed in FS relative to the SH condition. For the tannins, significant effects of light condition, harvest date and their interaction were observed. Contents of other compounds were not significantly affected by either light conditions or harvest dates.

Evolution of sucrose and reducing sugar (RS) contents was also measured in pericarp, endosperm and perisperm tissues of SH and FS fruits over the course of maturation. Irrespective of light treatments, the RS content of the pericarp was low from flowering to 175 DAF; it increased suddenly afterwards, reaching a higher value under SH than in FS conditions (404 vs. 326 mg g<sup>-1</sup> dry weight (DW), respectively) (Fig. 2A). Under both light conditions, sucrose content in the pericarp followed a similar trend, with a great increase after 175 DAF, reaching similar values (close to 85 mg g<sup>-1</sup> DW) at harvest (Fig. 2D). In the endosperm, high RS and low sucrose levels were observed under both light conditions between 120 and 175 DAF, which corresponds to the expansion phase of this tissue (Fig. 2B and E). After 197 DAF, RS content decreased gradually, becoming negligible by the mature stage, whereas sucrose content increased suddenly, reaching a peak (≈ 73 mg g<sup>-1</sup> DW) at 231 DAF under both FS and SH conditions. As in the pericarp, a slight but significant decline in sucrose content was observed during the last month of maturation under shade. In the perisperm, RS content was higher in SH than in FS conditions (Fig. 2C), while no major differences in sucrose contents were observed (Fig. 2F).

Table 2

Reducing sugar and sucrose contents in mature beans of *C. arabica* L. cv. IAPAR 59 under full-sun (FS) and shade (SH) conditions

Harvest time	Total sugars		Reducing sugars		Sucrose	
	FS	SH	FS	SH	FS	SH
214 DAF	8.79 ± 0.11	8.59 ± 0.15	0.33 ± 0.03	0.47 ± 0.01	8.46 ± 0.10	8.12 ± 0.16
281 DAF	8.70 ± 0.39	8.04 ± 0.56	0.19 ± 0.03	0.31 ± 0.13	8.51 ± 0.37	7.73 ± 0.64
<i>F</i> -value LC		4.47 <sup>ns</sup>		11.05*		6.59*
<i>F</i> -value HD		2.43 <sup>ns</sup>		14.02**		0.58 <sup>ns</sup>
<i>F</i> -value LC × HD		1.29 <sup>ns</sup>		0.09 <sup>ns</sup>		1.01 <sup>ns</sup>

For both conditions, two harvests were evaluated: 214 DAF and 281 DAF. Values are given in % of bean dry weight (DW). *F*-values are indicated for LC (light condition) and HD (harvest date) and LC × HD effects. <sup>ns</sup>*P* > 0.05, \**P* < 0.05, \*\**P* < 0.01.

### 3.4. Enzymatic activities

#### 3.4.1. Enzymatic activities in the pericarp tissue

SUS activity in the pericarp exhibited varying patterns depending on the light regime. In the FS condition, activity increased continuously from 60 to 197 DAF and diminished thereafter until the harvest (Fig. 3A). Under SH condition, two peaks of similar levels were observed at 144 and 197 DAF. At harvest, SUS activity was higher in the SH than in the FS conditions.

SPS activity profiles were similar to those of SUS (Fig. 3B). Maximum activities were observed at 197 DAF, with higher activities in the FS than in the SH condition. In both treatments, SPS activities diminished between 197 and 231 DAF. At harvest, higher SPS activity was observed in the SH than in the FS condition. Invertase profiles appeared similar during pericarp development under both light conditions, with peaks of activity of comparable amplitude (Fig. 3C). Under shade conditions, AI activity declined rapidly after 231 DAF, becoming undetectable at harvest. At this time, FS pericarp exhibited higher AI activity than those from SH.

#### 3.4.2. Enzymatic activities in the perisperm tissue

In both treatments, SUS activity was low during early developmental stages of the perisperm up to 120 DAF (Fig. 4A); activity increased thereafter, reaching a peak that had a higher value in the SH (197 DAF) than in the FS (175 DAF) conditions. In the shade, SUS decreased notably, down to 2.91 ± 0.22 μg sucrose μg protein<sup>-1</sup> h<sup>-1</sup> at 231 DAF. The activity profiles of SPS also followed those of SUS in both light regimes (Fig. 4B). Under shade, SPS activity reached a maximum value at 197 DAF which was twice that observed at 144 DAF under FS condition (Fig. 4B). After this point, SPS



Table 3  
Biochemical composition of dry beans of *C. arabica* L. cv. IAPAR 59 under full-sun (FS) and shade (SH) conditions

Harvest time	Caffeine <sup>a</sup>		Chlorogenic acids (CGA) <sup>a</sup>		Proteins <sup>a</sup>		Lipids <sup>b</sup>		Tannins <sup>a</sup>		Trigonelline <sup>b</sup>	
	FS	SH	FS	SH	FS	SH	FS	SH	FS	SH	FS	SH
214 DAF	1.32 ± 0.04	1.38 ± 0.09	6.55 ± 0.23	6.59 ± 0.86	14.82 ± 0.40	14.52 ± 0.74	15.36 ± 0.35	14.80 ± 0.10	4.34 ± 0.02	4.26 ± 0.05	0.92 ± 0.03	0.91 ± 0.01
281 DAF	1.24 ± 0.19	1.17 ± 0.20	5.78 ± 0.31	6.46 ± 0.28	14.38 ± 0.67	13.46 ± 0.77	15.23 ± 0.30	14.60 ± 0.17	3.84 ± 0.09	4.06 ± 0.11	0.88 ± 0.02	0.87 ± 0.01
<i>F</i> -value LC <sup>c</sup>	0.02 <sup>ns</sup>		1.57 <sup>ns</sup>		2.48 <sup>ns</sup>		16.83 <sup>**</sup>		2.47 <sup>ns</sup>		0.89 <sup>ns</sup>	
<i>F</i> -value HD <sup>c</sup>	2.94 <sup>ns</sup>		2.45 <sup>ns</sup>		3.79 <sup>ns</sup>		1.30 <sup>ns</sup>		58.32 <sup>***</sup>		9.39 <sup>*</sup>	
<i>F</i> -value LC × HD <sup>c</sup>	0.61 <sup>ns</sup>		1.36 <sup>ns</sup>		0.67 <sup>ns</sup>		0.05 <sup>ns</sup>		11.55 <sup>**</sup>		0 <sup>ns</sup>	

For both conditions, two harvests were evaluated: 214 DAF and 281 DAF. Values are given in % of bean dry weight (DW). CGA, chlorogenic acids.

<sup>a</sup> Values were obtained by biochemical analysis.

<sup>b</sup> Values were obtained by near infrared reflectance spectroscopy.

<sup>c</sup> *F*-values are indicated for LC (Light condition) and HD (Harvest date) and LC × HD effects. <sup>ns</sup>*P* > 0.05, <sup>\*</sup>*P* < 0.05, <sup>\*\*</sup>*P* < 0.01, <sup>\*\*\*</sup>*P* < 0.001.

activity declined steeply, down to a value of 231 DAF which was similar to that observed in FS at 175 DAF. AI activity was higher in the FS than in the SH conditions during early developmental stages; activity increased greatly between 144 and 231 DAF in SH (Fig. 4C). However, as long as it was possible to separate the perisperm tissue, AI activity always remained undetectable after 87 DAF in FS condition.

### 3.4.3. Enzymatic activities in endosperm tissue

Under both light conditions, SUS activity was low in young endosperm, but increased during later developmental stages (Fig. 5A). In the FS condition, SUS activity reached a maximum at 175 DAF and decreased at harvest. Under shade, SUS activity increased towards the end of maturation and appeared three-fold higher than in the FS condition. SPS activity exhibited a peak at 144 DAF and diminished thereafter until harvest under full sun (Fig. 5B). In contrast, SPS activity increased regularly between 120 and 197 DAF in SH endosperm, declined between 197 and 231 DAF and increased greatly thereafter, with the highest value being observed at harvest. Irrespective of the light conditions, AI activities during endosperm development were always very low (Fig. 5C).

### 3.5. Expression of *SUS* genes in endosperm of FS and SH coffee fruits

Light conditions modified steady-state levels of *SUS*-encoding genes in the endosperm (Fig. 6). Using probes specific for *CaSUS1* and *CaSUS2* mRNA from *C. arabica*, it was observed that *CaSUS1* transcripts were weakly detectable during early development (120 DAF); then levels strongly increased at 144 DAF and diminished thereafter, becoming undetectable at the harvest under FS condition. Under shade, maximum *CaSUS1* expression was detected at an early stage (at 120 DAF) than in FS, and then decreased gradually thereafter. In both light conditions, *CaSUS2* transcripts were barely detected during endosperm development and accumulated at maturation, reaching a higher level in the SH than in the FS condition.

## 4. Discussion

### 4.1. Effects of light regime on coffee fruit development

The present results confirmed the importance of light regime in controlling the final bean characteristics. We demonstrated that shade delayed the development and maturation of coffee fruits for at least 1 month, without affecting fruit FW, which was similar in both light regimes. At the tissue level, shade reduced pericarp FW and thickness (data not shown), but increased endosperm (bean) FW at harvest, as confirmed by the increase of approximately 10% of the bean weight and volume in the SH compared with the FS treatment. These values are in the same range as previously reported [27]. We also showed an inverse relationship between pericarp and endosperm FW with light regimes. The importance of pericarp

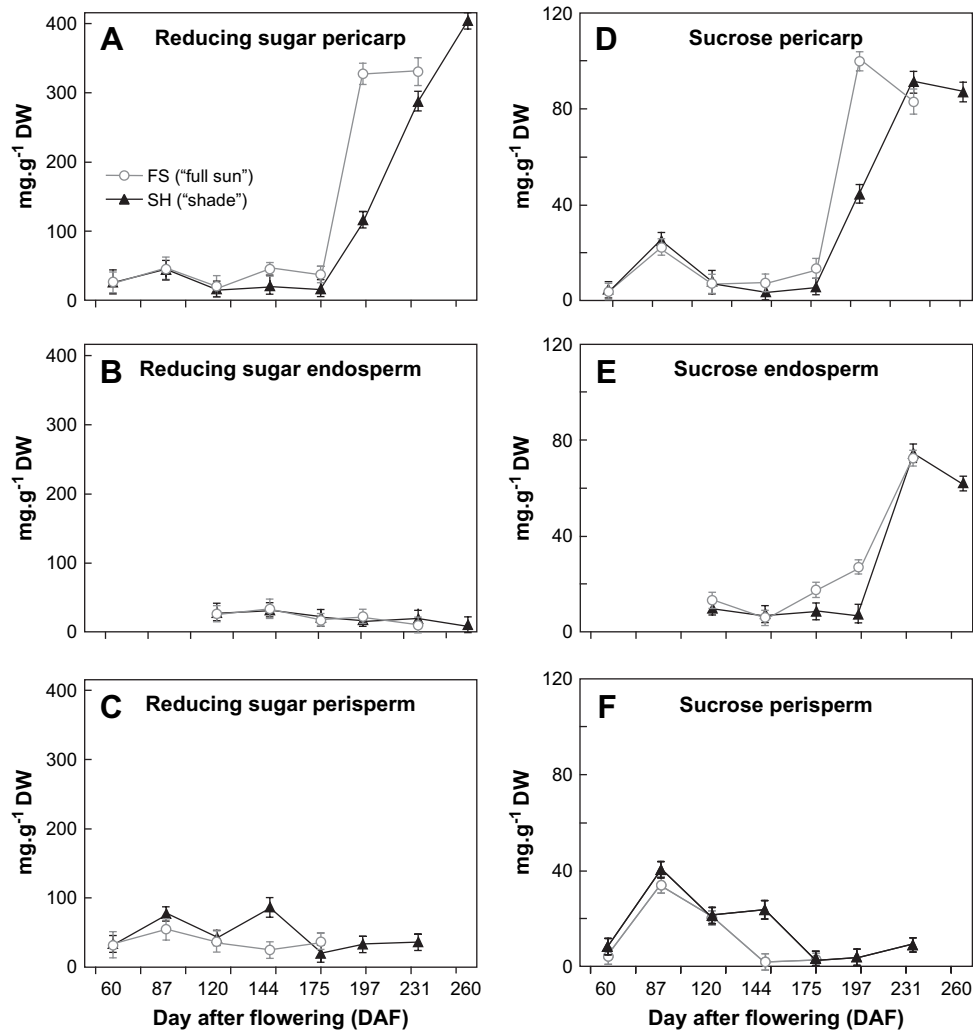


Fig. 2. Evolution of reducing sugars and sucrose contents in separated tissues from fruits of *C. arabica* L. cv. IAPAR 59 plants grown under full-sun (FS) and shade (SH). Data are expressed as mg g<sup>-1</sup> of dry weight (DW) for reducing sugars (A–C) and sucrose (D–F) in pericarp, perisperm and endosperm tissues. Open circles and closed triangles are for full-sun (FS) and shade (SH), respectively. Error bars indicate standard deviation (SD) of repetitions ( $n = 3$ ).

in coffee berry photosynthesis is far from negligible, especially in view of the large berry sink strength and the finding that the berry photosynthetic area represents up to 20% of the total tree photosynthetic area at the highest fruit load [41]. Even if leaf photosynthesis contributed more to seed development than to the pericarp in terms of photosynthate supply, pulse-chase experiments with <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>C-sugars also revealed the importance of the pericarp in terms of photosynthate supply to the endosperm [12]. Altogether, these results reinforce the idea that slowing down the ripening process by shade favors bean filling [42].

In plant seeds, high ratios of reducing sugar (RS) to sucrose (S) contents are characteristic of intensive cell division in young tissues [45]. The transition of the pre-storage phase to the maturation (storage) phase is then accompanied by a switch in carbohydrate state, from a high to low RS/S ratio. In coffee, the role of the perisperm, during the expansion, in determining the final size of the locular space, has been proposed [8,31]. Here, we have demonstrated that the perisperm is larger in

SH fruits than in FS fruits. This is probably a consequence of the maintenance of high RS/S ratios, thereby explaining the large bean size observed under the SH condition. At this time, we do not know if the increased size of the perisperm resulted from higher cell number, higher cell volume or a combination of both. The same question also applies to the endosperm. Because shade was artificially applied by a net installed over the plants one month after flowering, it is probable that the increase in bean size results from development of larger cell size rather than from an increase in cell number. Therefore, histological studies and detailed analyses of polysaccharide composition of SH and FS coffee fruits are worth undertaking to address this question.

#### 4.2. Effects of light regime on coffee fruit composition

Light experienced by coffee plants modified the final biochemical composition of fruits, as illustrated by the reduction in lipid content in SH dry beans compared to FS beans. This

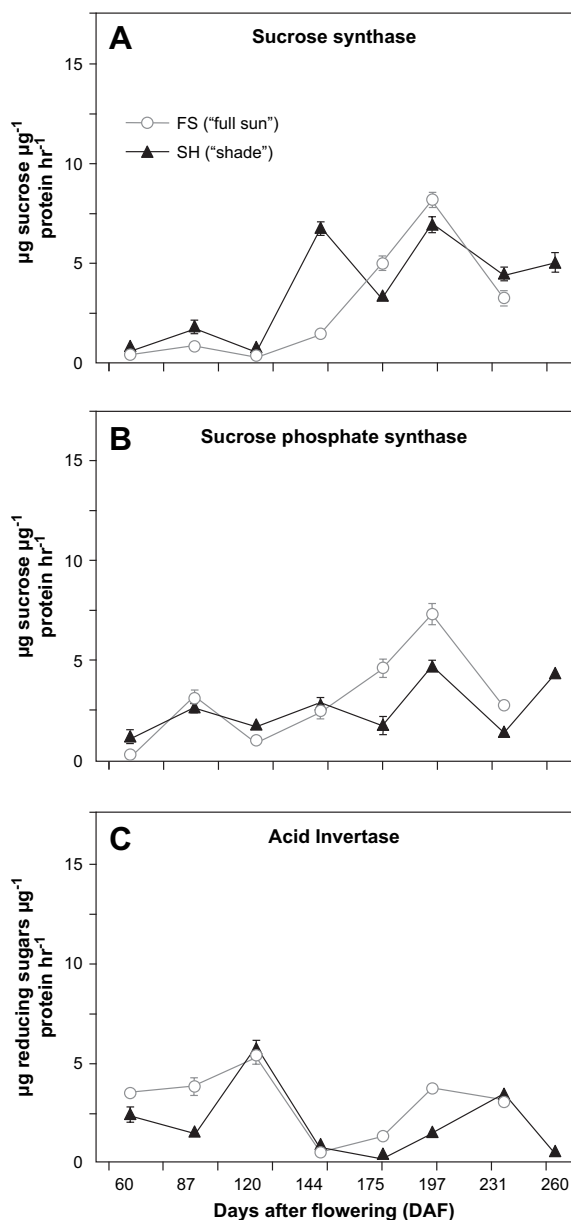


Fig. 3. Enzymatic activities in isolated pericarp tissues during ripening of *C. arabica* L. cv. IAPAR 59 fruits. Sucrose synthase (A) and sucrose-phosphate synthase (B) activities are expressed as  $\mu\text{g sucrose } \mu\text{g protein}^{-1} \text{ h}^{-1}$  during ripening. Acid invertase (C) activity is expressed as  $\mu\text{g reducing sugars } \mu\text{g protein}^{-1} \text{ h}^{-1}$ . Open circles and closed triangles are for full-sun (FS) and shade (SH), respectively. Error bars indicate standard deviation (SD) of repetitions ( $n = 3$ ).

result contrasts with those of previous studies, which showed a negative correlation between sucrose and lipid content in beans of *C. arabica* [4,9]. However, CGA, protein, tannin, trigonelline and caffeine contents did not differ significantly between light regimes. The quantification of additional biochemical compounds (e.g. free amino acids, peptides) of beans could be instrumental in obtaining an in-depth overview of shade effects on coffee bean metabolism.

Another effect of shade on fruit biochemical composition concerns sucrose content, which was lower in both pericarp

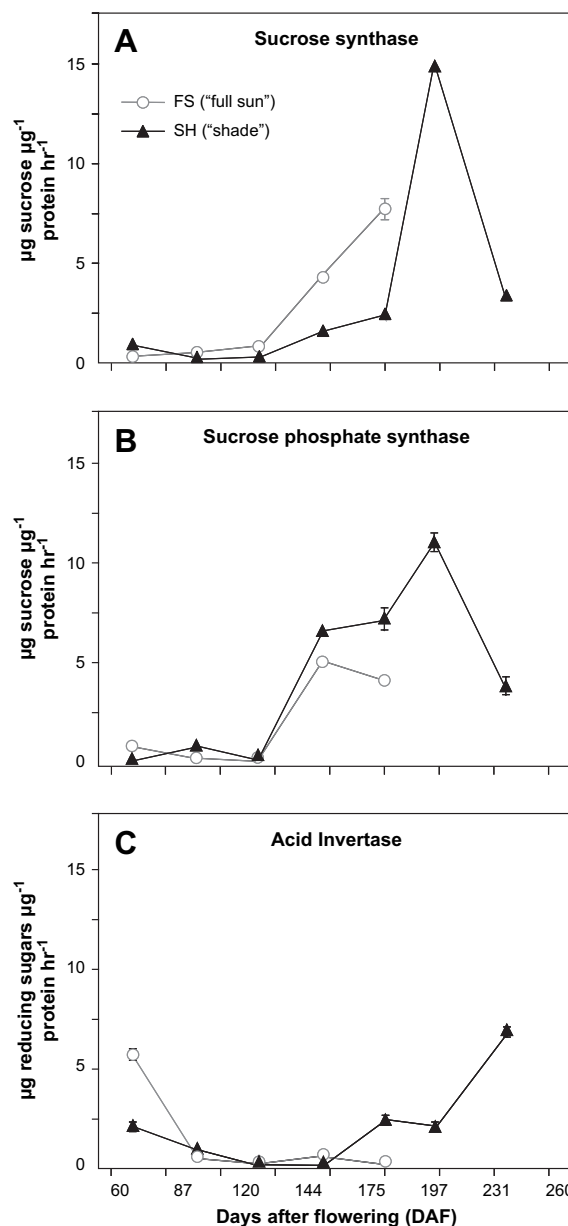


Fig. 4. Enzymatic activities in isolated perisperm tissues during ripening of *C. arabica* L. cv. IAPAR 59 fruits. Sucrose synthase (A) and sucrose-phosphate synthase (B) activities are expressed as  $\mu\text{g sucrose } \mu\text{g protein}^{-1} \text{ h}^{-1}$  during ripening. Acid invertase (C) activity is expressed as  $\mu\text{g reducing sugars } \mu\text{g protein}^{-1} \text{ h}^{-1}$ . Open circles and closed triangles are for full-sun (FS) and shade (SH), respectively. Error bars indicate standard deviation (SD) of repetitions ( $n = 3$ ).

and endosperm tissues at maturation in plants grown in SH than in those grown under FS condition, whereas the opposite was observed for reducing sugars. The present data agree with those of Vaast et al. [42] who reported a reduction in sucrose content in the shade environment in beans of *C. arabica* Costa Rica 95 (Catimor) cultivar but contrast with the results observed with the Catuai cultivar, in which a higher sucrose content was observed in beans grown in the shade compared to those grown in full-sun conditions [17]. These discrepancies could be explained by the fact that coffee cultivars respond



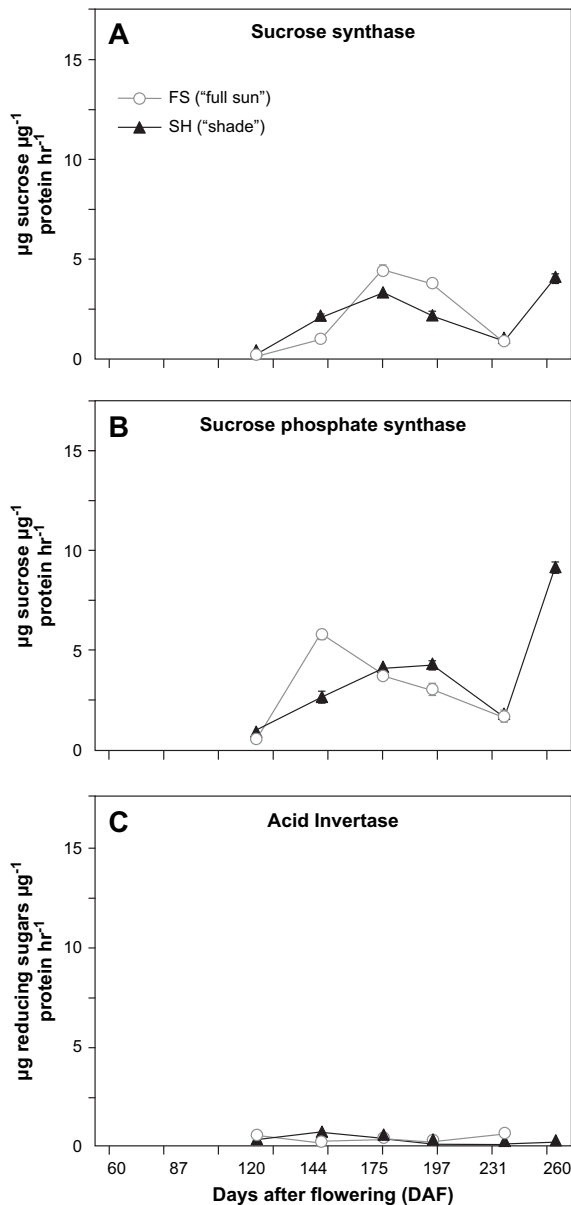


Fig. 5. Enzymatic activities in isolated endosperm tissues during ripening of *C. arabica* L. cv. IAPAR 59 fruits. Sucrose synthase (A) and sucrose-phosphate synthase (B) activities are expressed as  $\mu\text{g sucrose } \mu\text{g protein}^{-1} \text{ h}^{-1}$  during ripening. Acid invertase (C) activity is expressed as  $\mu\text{g reducing sugars } \mu\text{g protein}^{-1} \text{ h}^{-1}$ . Open circles and closed triangles are for full-sun (FS) and shade (SH), respectively. Error bars indicate standard deviation (SD) of repetitions ( $n = 3$ ).

in different ways to similar environmental conditions. In that case, it is worth noting that both IAPAR 59 (this study) and Costa Rica 95 cultivars contain introgressed genome from *C. canephora* via the Timor hybrid while Catuai corresponds to a non-introgressed cultivar of *C. arabica* [37]. In order to know if the reduction in sucrose content in the shade environment observed in introgressed materials (Sarchimor and Catimor) is really an inheritance of *C. canephora*, it should be worth analyzing the effects of light regime on sucrose metabolism in the latter species.

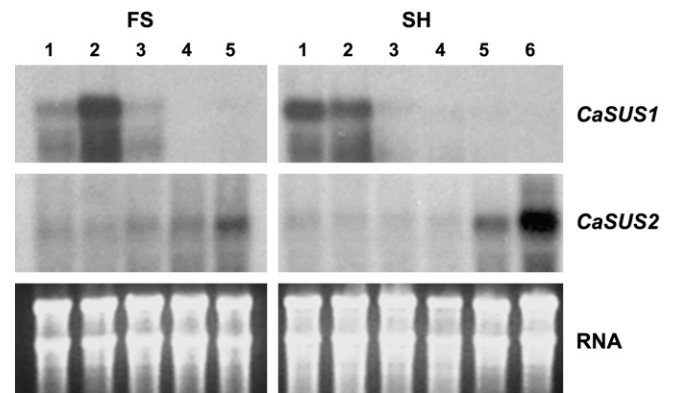


Fig. 6. Expression of *CaSUS1* and *CaSUS2* genes during endosperm development of fruits grown in full-sun (FS) and shade (SH) conditions. Total RNA isolated from endosperm was separated on a formaldehyde-agarose gel, transferred onto a nylon membrane and hybridized independently with *CaSUS1* and *CaSUS2* probes at regular developmental stages: lane 1, 120 DAF; lane 2, 144 DAF; lane 3, 175 DAF; lane 4, 197 DAF; lane 5, 231 DAF and lane 6, 260 DAF (only in SH conditions). rRNAs stained by ethidium bromide were used to monitor the equal loading of RNA samples.

It is also known that topography (slope exposure, altitude) and meso-climatic conditions (air temperature and hygrometry) modify bean chemical composition [4,36]. Some of the differences reported here could also be explained by post-harvest treatments. For example, Knopp et al. [21] reported that unwashed (dry process, as used in this study) coffee beans usually had higher fructose and glucose contents than those treated by the wet process commonly used in Central America. Together, these considerations make difficult the comparison of beans treated by different post-harvest processes and harvested from plants grown in different climatic conditions.

#### 4.3. Effects of light regime on enzymatic activities

Although preliminary, the present results show that activities of several sucrose-metabolizing enzymes are modified by SH conditions. This is illustrated in mature pericarp and the perisperm, where peaks of SUS and SPS activities were higher in plants grown in SH than in FS conditions. The same was also observed for acid invertase (AI) in the last stages of perisperm. These results also highlight the difficulty in determining clear relationships between changes measured enzymatically in separated tissues and those concerning sugar (reducing sugars and sucrose) contents. For example, high SPS and SUS activities observed in shaded endosperm were not paralleled by a subsequent increase in sucrose content in this tissue. The same was observed for AI activity in shaded perisperm; a concomitant increase in reducing sugars was not observed. In contrast, the negligible AI activity in the shaded endosperm does not explain the increase in reducing sugars in this tissue before the harvest. Such observations, previously reported by Geromel et al. [12], were attributed to intense sucrose degradation, (re)synthesis and transfers occurring between the pericarp, perisperm and endosperm of coffee fruits. However, the action of other sucrose-cleaving enzymes

[19,39], the regulation of sucrose synthase by sub-cellular localization and the presence of enzymatic activities in cell compartments are difficult to investigate (e.g. apoplastically localized) [28] and cannot be completely ruled out. Nonetheless, the high enzymatic activities retained in the perisperm confirmed the importance of this tissue in controlling coffee bean development [8,31].

The present results also significantly contrast with those previously reported on the same plants grown under FS conditions a year before [12]. For example, SUS activities at harvest in the pericarp and endosperm were two times lower here than those reported in 2002–2003. Alternate bearing of coffee trees, with low and high productivity observed for these plants in 2002–2003 (mean 2340 kg dried coffee beans ha<sup>-1</sup>) and 2003–2004 (mean 3400 kg ha<sup>-1</sup>), respectively, could explain part of these discrepancies [6]. Environmental factors such as water deficits were reported to alter both the activity and expression of SUS. In addition, in many plant systems where SUS is thought to be involved in meeting the glycolytic demand during water stress, its gene expression and activity are usually increased under drought conditions. This was reported in the leaves of *Populus popularis* [29] and *Arabidopsis thaliana* [10]. In contrast, reduced SUS activity and protein content were also reported in response to moderate water stress in soybean nodules [14]. The occurrence of a dry season in 2003–2004 (between 197 and 231 DAF, data not shown) could explain the rapid decline of SUS and SPS activities in pericarp, perisperm and endosperm tissues. Even though physiological analyses (water potential status, leaf net photosynthesis) were not performed during this dry season, we did not observe any noticeable alterations in tissue FW or reducing sugar/sucrose content during this period. This suggests that water deficit had only limited effects on the accumulated sugar content amassed in coffee fruit tissues.

#### 4.4. Effects of light regimes on expression of SUS genes

Shade quantitatively and temporally modifies the expression of SUS-encoding genes in coffee beans. In *C. arabica*, this enzyme is encoded by two different genes, *CaSUS1* and *CaSUS2*, which are differentially expressed during endosperm development [12,24]. *CaSUS1* transcripts accumulate mainly during the early development of endosperm; whereas *CaSUS2* transcript levels parallel sucrose synthase activity during the final maturation. The results also clearly show an earlier expression of *CaSUS1* and a higher expression of *CaSUS2* at maturation in SH as opposed to FS conditions. It is also worth noting that SUS mRNA and protein levels were not closely coupled, as illustrated here by the fact that the maximum *SUS1* expression was detected before the peak of SUS activity, which was usually observed at 175 DAF. Such observations were previously reported for SUS during fruit development of *C. racemosa* [13], but also for other plants, such as tomato [44]. This could be attributed to particular regulation processes commonly reported to occur for SUS [46]. For example, it is possible that the accumulated *SUS1* transcripts were not translated, as reported for the *Sh* gene, which encodes an

SS1 isoenzyme of SUS in maize seedlings subjected to anaerobic stress [40]. Because *SUS1* protein was clearly identified in the endosperm at 118–147 DAF by Western blot analysis [12], this possibility is unlikely. Another explanation is that *SUS1* could require post-translational modifications in order to be activated, as previously reported in soybeans [33]. *SUS* protein is also subjected to reversible phosphorylation that controls its subcellular localization (between the cytosol, plasma membrane and actin cytoskeleton) and consequently activity [46]. Such post-translational modifications do not seem to occur for the *CaSUS2* isoform because its transcript paralleled sucrose synthase activity in the last weeks of endosperm development under SH conditions.

## 5. Conclusion

The present work constitutes the first study detailing the effects of shade on tissue development, sugar metabolism and gene expression in coffee fruits. It clearly demonstrates a relationship between the greater perisperm development occurring early in fruit development and the larger size of mature beans commonly observed under SH conditions. The enhanced *SUS* activity detected in mature endosperm under SH conditions, compared with FS, was attributed to the higher expression of *CaSUS2* gene. However, these changes were not accompanied by a concomitant increase in sucrose content in mature beans which appeared lower in SH than in FS conditions, suggesting a redirection of sucrose metabolism in other metabolic pathways (e.g. complex polysaccharides) that needs to be further identified.

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